

# Potential salivary biomarkers and their genetic effects in a pilot study of adolescent boys with externalizing problems

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**Aims:** Beside the well-known stress response marker cortisol, salivary alpha-amylase is receiving increasing attention. Numerous studies have investigated the potential biomarker properties of cortisol mirroring abnormal hypothalamic-pituitary-adrenal axis activity in connection to both internalizing and externalizing behavior problems. The other major physiological system involved in stress reactivity, the sympathetic nervous system activity can be also measured by the surrogate marker of salivary alpha-amylase. Most of the studies applied a stressful situation to obtain inter-individual differences in stress-reactivity, although differences in the baseline level of cortisol have been also shown in relation to externalizing problems. To test the relevance of another (easier) biomarker, we selected to study baseline circadian salivary cortisol and alpha-amylase levels among adolescent boys with externalizing problems. **Methods:** Saliva samples were collected at 3 time-points (morning, noon, evening) during 3 consecutive days from 37 inpatient boys (mean age  $12.4 \pm 1.0$ ). Cortisol and alpha-amylase levels were measured by enzyme-linked immunosorbent and kinetic enzyme assays, respectively. Genetic variants in the hypothalamic-pituitary-adrenal axis and the norepinephrine transporter or catecholamine metabolizing enzymes were tested for potential moderating effects at these salivary biomarkers. **Results:** Saliva cortisol showed the classical diurnal fluctuation in boys with externalizing problems (possibly from a lower morning level), but it was not modified by the presence of either conduct, oppositional defiant or attention-deficit/hyperactivity disorder. The diurnal fluctuation of the salivary alpha-amylase levels was also typical, but the presence of conduct disorder was associated with significantly lower alpha-amylase activity ( $p=0.024$ ) among boys with externalizing problems. The catechol-O-methyltransferase Val158Met (rs4680) polymorphism had an additional effect on salivary alpha-amylase: boys with homozygote genotypes had lower alpha-amylase activity at all 3 time-points compared to Val/Met heterozygotes ( $p=0.045$ ). **Conclusions:** Our preliminary data suggest that salivary alpha-amylase might be used to further characterize subgroups within externalizing problems, however, this biomarker might be modified by certain genetic polymorphisms.

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**Keywords:** alpha-amylase, conduct disorder, cortisol, externalizing behavior, stress

The two major physiological systems that mediate stress responses to environmental challenges are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Chrousos, 2009). Stimulation of the HPA axis leads to secretion of cortisol from the adrenal cortex to the blood stream

where it is transferred by corticosteroid-binding globulin throughout the body (this represent the bound fraction). As a steroid molecule, cortisol enters saliva by passive diffusion from the capillaries surrounding the salivary glands. Importantly, salivary cortisol reflects the active (free) cortisol level, hence it is the

preferred biological sample for stress-reactivity over the last decades (Hellhammer et al., 2009). Another recently proposed salivary analyte, alpha-amylase (AA) reflects the concentration of norepinephrine caused by SNS modulations (Granger et al., 2007; Nater and Rohleder, 2009). Since AA is a digestive enzyme that breaks down starch into soluble maltose and dextrin, it can be measured quickly and reproducibly with substrates that utilize its enzymatic activity (expressed as units per milliliter (U/ml), Bosch, 2011). Therefore, stress-mediated activation of the HPA axis and the SNS can be measured in non-invasively collected saliva samples (via cortisol level and AA activity, respectively) among children or even infants (see for examples Adam et al., 2011; Rash et al., 2016).

Cortisol and AA can be studied under basal condition or in psychosocial stress situations. Circadian activity of cortisol is characterized by the cortisol awakening response and a decline to the nadir around midnight, whereas salivary AA (sAA) exhibits an opposite pattern of circadian activity with a low level at waking that gradually increases through the day (Nater et al., 2007). During a psychosocial stress test cortisol shows a slow increase and returns to baseline slowly (~ 40 min), whereas sAA has a rapid increase and recovers to the baseline faster (~ 10 min), which corresponds well to the slower hormone response of cortisol vs. fast SNS activity (Nater et al., 2005; Ali and Pruessner, 2012).

Repeated stress situations (such as maltreatment) in early childhood have been shown to alter stress response systems, and result in atypical HPA axis activity (Cicchetti and Rogosch, 2001; van der Vegt et al., 2010). The accompanying psychopathologies include both internalizing problems (with higher cortisol levels) and externalizing problems (with flattened cortisol pattern or lower cortisol reactivity) (Cicchetti and Rogosch, 2001; Hagan et al., 2014). According to DSM-IV-TR and DSM-5, disruptive problems in children and adolescents might involve attention-deficit/hyperactivity disorder (ADHD), oppositional defiant disorder (ODD) and most importantly, in relation with later antisocial personality disorder, conduct disorder (CD). Previous studies indicated lower basal or stress-related salivary cortisol levels in different groups of children and adolescents with disruptive problems (disruptive problem undifferentiated: McBurnett et al., 2000; de Vries-Bouw et al., 2012; ODD: van Goozen et al., 1998; ODD and ADHD: Karyawasam et al., 2002; CD: Fairchild et al., 2008; Pajer et al., 2001; Vanyukov et al., 1993, von Polier et al., 2012). In one of the studies basal salivary cortisol

levels did not differ between boys with ADHD alone compared to ADHD with comorbid CD, however, their stress-reactivity was different (Northover et al., 2016). These results were not always replicated, and controversial results also emerged (Schulz et al., 1997; Bae et al., 2015; Susman et al., 2010). Interestingly, these latter two studies showed major differences between cortisol and sAA response levels (Bae et al., 2015; Susman et al., 2010).

In our present pilot study, we compared the diurnal cortisol and sAA patterns in children with externalizing problems, but our focus was somewhat different compared with earlier studies. Our major intention was to compare children with certain types of externalizing problems, e.g. according to the presence or absence of CD or ODD. Only boys were included in our study in order to reduce heterogeneity, because of the fluctuating hormonal status of adolescent girls and the apparent sex differences in cortisol levels (see for examples, Shirtcliff et al., 2005; Marsman et al., 2008).

In addition, we checked possible genetic influence on the stress-related biomarkers, such as those single nucleotide polymorphisms (SNPs) in the glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2) genes with potential functional relevance (DeRijk, 2009). Also, childhood trauma associated protective haplotype of the corticotropin releasing hormone receptor 1 (CRHR1) gene was selected (combination of rs110402, rs7209436 and rs242924, Polanczyk et al., 2009). To assess genetic influence on the SNS activity, previously indicated SNPs of the norepinephrine transporter gene (NET, SLC6A2), and two common functional polymorphisms in the metabolizing enzyme genes (monoamine oxidase A 30 bp VNTR and catechol-O-methyltransferase Val158Met, rs4680) were selected (Gizer et al., 2009).

## METHODS

### Participants

The study was designed in compliance with the Helsinki Declaration and was approved by the Local Scientific and Research Ethics Committee of the Medical Research Council. Patients receiving 5 day treatment at the Vadaskert Child Psychiatry Hospital and their parents provided written informed consent for their participation. Originally, we aimed to recruit Caucasian adolescent boys (in the age range of 10-14 years) with externalizing problems, and

**Table 1** Clinical characteristics of adolescent boys

		total (n=37)		CD- (n=21)		CD+ (n=16)		Statistics
		n	freq (%)	n	freq (%)	n	freq (%)	p-value
DSM-IV. diagnosis	dysthymia	16	43.2	10	47.6	6	37.5	n.s.
	ADHD	25	67.6	13	81.3	12	75.0	n.s.
	ODD	24	64.9	9	42.9	15	93.8	0.001
	CD	16	43.2	0	0.0	16	100.0	-
Age (mean±SD)		12.4±0.96		12.6±0.94		12.2±1.04		n.s.

DSM-IV. diagnostic groups are listed for those with  $n > 5$  cases in the total group. Abbreviation are ADHD: attention-deficit/hyperactivity disorder, CD: conduct disorder, ODD: oppositional defiant disorder.

a comparison in relation with the presence of conduct disorder (CD) was planned. However, the population of boys with externalizing problems had a high level of comorbidity (ADHD, ODD, or dysthymia). Exclusion criteria were (i) developmental indication of IQ lower than 70, (ii) psychotic condition, (iii) pervasive developmental disorder. Diagnoses were established by a child psychiatrist based on the Hungarian child version of the M.I.N.I. – International Neuropsychiatric Interview (M.I.N.I. Kid). The M.I.N.I. Kid is a validated structured diagnostic interview determining 22 child-psychiatric diagnoses according to DSM-IV criteria, and a validated Hungarian version was also provided (Balazs et al., 2004).

Among the 37 boys with externalizing problems, dysthymia, ADHD, ODD and CD was present in an overlapping pattern (Table 1). As the presence of CD was the main target, those children with or without CD were compared. The proportion of ADHD and dysthymia did not differ between the CD+ vs CD- groups but those with CD had higher proportion of ODD. Neither the age nor socioeconomic background was different between the two groups.

### Salivary measurements

Saliva samples were collected using passive drooling technique at the inpatient unit 3 times a day before the main meals: 8 a.m. (before breakfast), at noon (before lunch) and at 7.30 p.m. (before dinner) in three consecutive days (Tuesday-Wednesday-Thursday). Samples were stored at -20°C until shipment to the laboratory. After the first thawing of the samples, saliva (1-3 ml total volume) was centrifuged (2000 g,

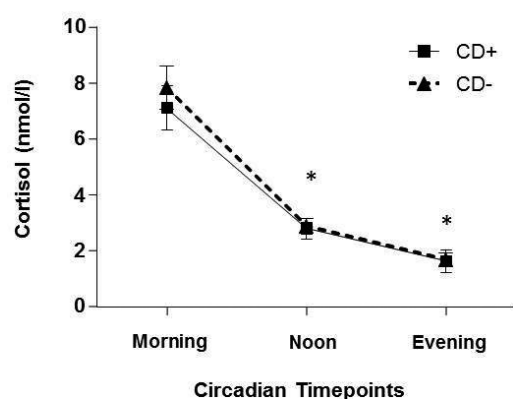
10 min) and the supernatant was aliquoted to (3-5 times) 0.25 ml volume for the measurement of cortisol concentration and AA activity. Salivary cortisol was measured with Salimetrics ELISA kit (cat. 1-3002) and sAA was measured with Salimetrics kinetic assay kit (cat. 1-1902) at the Laboratory of Molecular Neuroendocrinology which is approved by Salimetrics (Carlsbad, CA, USA) as a Center of Excellence Laboratory in Budapest, Hungary. All assays had technical duplicates according to the manufacturer's instructions, the mean values of the 2 measurements were used in the statistical analyses.

### Isolation of DNA and genotyping

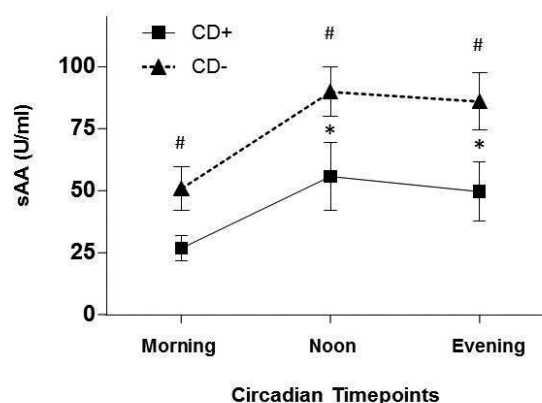
Genomic DNA was isolated from the cell pellet of the saliva samples with DNA purification kit obtained from Gentra (Minneapolis, USA). The HPA-axis related and NET SNPs were genotyped with pre-designed TaqMan probes (CRHR1 rs110402: C\_\_2544843\_10, rs7209436: C\_\_1570087\_10, rs242924: C\_\_2257689\_10; NR3C1 exon 9, rs6198: C\_\_8951023\_10; NR3C2 -2G/C, rs2070951: C\_\_1594392\_10; NET rs28386840: C\_\_60398891\_10, rs2242446: C\_\_26354911\_10, rs3785143: C\_\_27481932\_10, rs3785157: C\_\_27481947\_10, rs5569: C\_\_3020068\_10, rs7194256: C\_\_29079520\_10) on 7300 Real-Time PCR System (Applied BioSystem, Foster City, USA). The monoaminergic polymorphisms were genotyped following published protocols (Sabol et al., 1998; Tarnok et al., 2007). No significant deviations from Hardy-Weinberg equilibrium ( $p > 0.05$ ) were detected for any of the polymorphisms.

**Figure 1.** Salivary cortisol concentration (1.A: nM, mean $\pm$ SEM) and alpha-amylase activity (1.B: IU/ml, mean $\pm$ SEM) in boys diagnosed with externalizing disorders. CD+: conduct disorder is present (n=16), CD-: conduct disorder is not present (n=21). The asterisk denotes significant difference from the morning values ( $p<0.05$ ), whereas # show significant difference between the diagnostic groups in the GLM analysis.

**Figure 1.A.**



**Figure 1.B.**



### Statistical analyses

Statistica 7.0 software was used to analyze pooled diurnal cortisol and sAA data. A General Linear Model (GLM) was used to test the factorial effect of the presence of DSM-IV diagnosis (dysthymia, ADHD, ODD, CD) and the effect of time (in a repeated design, morning, noon and evening data) was also performed. The level of significance was set at  $p=0.05$ .

## RESULTS

Explorative analyses of salivary biomarker measures (using the mean values of the 3 days of each time points) were carried out separately in two comparison settings: based on presence / absence of DSM-IV diagnosis and of genetic risk variant. In the GLM analyses of salivary cortisol levels, only a major effect of time was present ( $F=96.627$ ,  $p<0.0001$ ) but diagnostic group effects (the presence of dysthymia, ADHD, CD, or ODD) was not detected, and interaction between time and group did not occur either. With Newman-Keuls post hoc comparison, noon and evening salivary cortisol data were significantly lower than morning values (Fig.1.A.).

In the sAA levels, additional to the time effect ( $F=19.978$ ,  $p<0.0001$ ), a significant difference was observed between CD- and CD+ groups ( $F=5.530$ ,  $p=0.024$ ) at each time points (Fig.1.B.). Interaction

between the time and group was not present. With Newman-Keuls post hoc comparison, noon and evening sAA values were significantly higher than morning values. Similar picture emerged when checking the ODD- vs ODD+ groups but the difference was only tendentious ( $F=4.072$ ,  $p=0.052$ ), and this effect is potentially caused by the substantial overlap of CD and ODD diagnoses (see Table 1). In the case of dysthymia and ADHD, the presence of the diagnosis did not cause any significant effect.

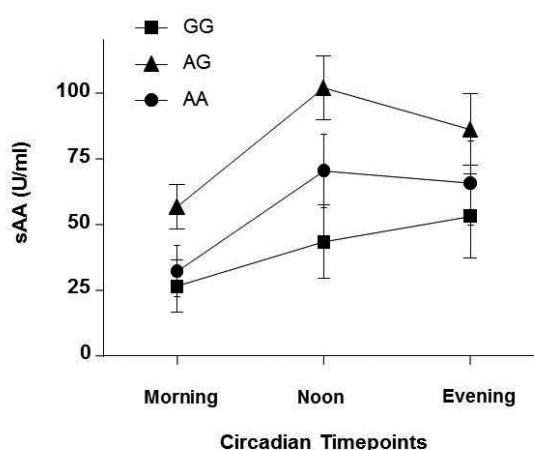
When checking the possible genetic effects on salivary biomarkers, only at sAA we could observe association with COMT Val158Met rs4680 (Figure 2.,  $F=3.389$ ,  $p=0.045$ ). There was no interaction between the genetic and diagnostic groups. Importantly, the genotype frequencies of this SNP did not differ between diagnostic groups, so we can conclude that CD and catecholamine polymorphism influenced sAA independently. We have to note that this nominally significant difference would not survive the correction for multiple testing, just warrant for possible genetic influence at sAA activity measurements.

## DISCUSSION

The main findings of the present preliminary study were the followings: First, saliva basal diurnal cortisol values did not differ in adolescent boys with externalizing problems according to the presence of conduct

**Figure 2.** Genetic effect of catecholamine polymorphisms on salivary alpha-amylase activity (IU/ml, mean $\pm$ SEM) in boys diagnosed with externalizing disorders. The 3 genotype groups of the catechol-O-methyltransferase Val158Met (rs4680) SNP are shown by the following symbols: circles = A/A = Met/Met homozygotes (n=11), triangles = A/G = Val/Met heterozygotes (n=15), squares = G/G = Val/Val homozygotes (n=11).

**Figure 2.**



disorder, but a major diurnal variation was observed. Second, sAA levels were significantly lower at each time-points among boys with CD compared to the boys with externalizing problems but without CD (which were in the same range as controls' sAA levels reported by Adam et al., 2011). Third, the diurnal fluctuation of both salivary cortisol and AA values were similar to that of previously reported child and adolescent data (Bae et al., 2015; Adam et al., 2011).

Salivary cortisol has been frequently measured as an indicator of HPA axis activity. The availability of another salivary marker reflecting SNS activity allows parallel measurement of the two major stress-responsive physiological systems. There have been a number of studies using these two salivary biomarkers simultaneously in stress-evoking test situations. However, according to our best knowledge, this is the first report on basal diurnal sAA level alterations in relation to CD diagnosis. Many of the previous studies did not separate this diagnostic group from other externalizing behavior problems. For example, Bae et al. (2015) analyzed children with either internalizing or with externalizing disorder compared to healthy control group. Our finding is in accordance with previous observations of sAA changes in adolescent and young adult at-risk populations (Kliewer et al., 2012; de Vries-Bouw et al., 2012). Lower salivary AA and cortisol levels were reported in 18-year-old delinquent males with disruptive behavior disorder compared

to the control group and these biomarkers showed significant inverse associations with dimensional measures of disruptive behavior (de Vries-Bouw et al., 2012). In a longitudinal study on peer aggression (assessed among 14-year old African Americans), the aggressors showed a decrease in sAA during the Social Competence Interview compared to the control group (which did not show any change) (Kliewer et al., 2012).

Limitations of our study include the relatively small sample size and the lack of control group. We carried out preliminary analyses with clinical orientation. The acquisition of similar data in control adolescents without clinical monitoring is a rather difficult one. Still, some publications could use similar measures in control adolescents as well (Bae et al., 2015; Susman et al., 2010), and in order to further clarify the importance of the measured variables, control data should also be acquired. At this point we can only speculate that basal salivary (morning) cortisol level of boys with externalizing behavior problems might be lower than that of controls by comparing our data to the published one using the same cortisol assay kit (Adam et al., 2011). As a targeted decision, only boys were included in the study, but the inclusion of girls might have a major importance as well (like Pajer et al., 2001).

As a conclusion, we outline the importance of parallel data collection of peripheral markers of the



stress-response system in children and adolescents with externalizing problems. It seems that the clinical subdivisions of externalizing problems in boys might relate to differences in peripheral stress markers, and that suggest a central alteration as well.

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## Externalizációs problémákkal küzdő serdülő fiúk nyál biomarkereinek azonosítása és ezek genetikai befolyásoltsága

**Célkitűzés:** A jól ismert stresszmarker kortizol mellett a nyál alfa-amiláz enzimje is egyre nagyobb figyelmet kap napjainkban. Számos tanulmány vizsgálta a kortizol mint potenciális biomarker tulajdonságait externalizáló és internalizáló viselkedési problémákban a hipotalamusz-hipofízis-mellékvese tengely kóros működésével kapcsolatosan. A stressz válaszreakcióban szerepet játszó másik fiziológiai rendszer, a szimpatikus idegrendszer működése vizsgálható a nyál alfa-amiláz enzimen keresztül. Az eddigi tanulmányok többsége teszt-szituációban vizsgálta a stresszreaktivitás egyéni különbségeit, bár externalizáló zavarok esetében a kortizol-alapszintekben is megfigyeltek különbségeket. Egy egyszerűbb biomarker tesztelésére nyál kortizol- és alfa-amiláz alapszinteket tanulmányoztunk externalizáló viselkedési problémákkal küzdő serdülő fiúk csoportjában. **Módszer:** A nyálmintákat három időpontban (reggel, délben, este), három egymást követő napon gyűjtöttük 37 bent fekvő fiú betegtől (átlagéletkor  $12,4 \pm 1,0$ ). A kortizol- és alfa-amiláz-szinteket enzim-kapcsolt immunszorbens, illetve kinetikus enzimreakción alapuló kitékkel mértük. A hipotalamusz-hipofízis-mellékvese tengely, a noradrenalin transzporter, valamint a katekolamin metabolizáló enzimek genetikai variánsainak lehetséges módosító hatásait is vizsgáltuk e két nyálbiomarker tekintetében. **Eredmények:** A nyál kortizolszintje a klasszikus napszaki ingadozást mutatta az externalizáló problémákkal küzdő fiúk körében, és nem mutatott eltérést sem a figyelemhiányos hiperaktivitási, sem az oppozíciós, sem a viselkedészavar tekintetében. A nyál alfa-amiláz-szintje szintén tipikus napszaki ingadozást mutatott, viszont a viselkedészavar jelenléte szignifikánsan alacsonyabb alfa-amiláz aktivitást eredményezett ( $p=0,024$ ) az externalizáló problémákkal küzdő fiúk körében. A katekol-O-metiltranszferáz Val158Met (rs4680) polimorfizmusa szintén befolyásolta az alfa-amiláz szintet: a homozigóta fiúk mindhárom vizsgálati pontban alacsonyabb alfa-amiláz aktivitást mutattak, mint a Val/Met heterozigóták ( $p=0,045$ ). **Következtetés:** Előzetes adataink arra utalnak, hogy a nyálból mérhető alfa-amiláz szintek alapján elkülöníthetők egyes alcsoportok az externalizáló viselkedés problémákon belül, azonban ezt a biomarkert egyes genetikai polimorfizmusok is befolyásolhatják.

**Kulcsszavak:** alfa-amiláz, viselkedészavar, kortizol, externalizációs zavarok, stressz